# Instructions for Use for Biofortuna SSPGo™ HLA Typing Kits

## CE Revision 5, January 2014

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## 1. Intended Use

Biofortuna HLA SSPGo Kits are qualitative DNA-based kits for determining HLA alleles in either 'low resolution' kits, or group-specific amplification of alleles in 'intermediate /medium level' resolution kits. The common definition of medium level resolution is where the majority of results are clearly defined at the two digit level; e.g. DQB1*02, DQB1*05. High resolution is generally defined as the majority of alleles identified are defined at the four-digit level such as DQB1*02:01, DQB1*05:01. This is an *in vitro* diagnostic product intended for use by trained personnel only.

## 2. Introduction

HLA molecules play a key role in immunity and recognition of self versus non-self, consequently HLA genotyping and HLA matching is mandatory prior to most forms of transplantation. As HLA antigens restrict the specificity of T-cell mediated immune responses HLA genotyping is a useful investigative tool in any immune disorder or any immune response to pathogens, vaccines or medical treatment. HLA genotyping can also be used to support disease diagnosis where certain HLA alleles have been shown to be significantly associated with disease states.

Most HLA genes are highly polymorphic and generally DNA genotyping is required for accurate determination of HLA antigens. PCR genotyping using Sequence-Specific Primers (SSP)[1], is a rapid method of HLA genotyping, particularly suitable for situations where medium level resolution is required. Biofortuna SSPGo kits all feature complete dried reactions, including polymerase, so that all the user has to do prior to PCR is add DNA.

Every effort is made to keep the kits updated with new IMGT HLA alignment releases. Kit updates are available from www.biofortuna.com.

## 3. Test Description

PCR SSP is based on the principle that only primers with completely matched 3' terminals to a target sequence will amplify. Mismatched primers do not yield positive amplification products[2]. An internal control primer pair, which amplifies a conserved region of a housekeeping gene, is included in every PCR reaction mix; the internal control primer pair is an indicator of the integrity of the PCR reaction. SSP genotyping generally uses multiple reactions that when analysed together indicate the genotype. Visualisation of the amplified products can be achieved using agarose gel electrophoresis systems which separate the DNA fragments by size.

## 4. Kit Contents

- Polypropylene PCR plates or strips consisting of between 8 and 96 PCR wells (kit dependant), each well containing pre-dispensed freeze dried primers, polymerase, dNTPs* and buffer. Each plate or strip is provided sealed with a sheet or cap and individually packed within a foil pouch containing a desiccant bag.
- PCR sealing sheets or caps
- 1x Instructions for use.
- Certificate of analysis
- Interpretation tables and MSDS can be downloaded from the Biofortuna website www.biofortuna.com. If you are unable to download from the website please contact your local distributor.

*CleanAmp™ dNTPs are licensed from Trilink Biotechnologies Inc for use in Biofortuna SSPGo products.

## 5. Reagents and Equipment Not Supplied

- Appropriate calibrated pipettors and sterile tips e.g. P10 pipettor with 10µl filter tips.
- DNA isolation kit/equipment.
- UV spectrophotometer.
- Polypropylene tubes.
- Sterile molecular grade water.
- A thermal cycler with the following specifications should be used:
  - 96 well thermal cycler with heated lid with a temperature of 104°C for oil-free operation
  - Ramp rate of 1.0°C/sec.
  - Temperature range of 4.0°C to 99.9°C
  - Temperature accuracy of ±0.25°C for the range of 35°C to 99.9°C
  - Temperature calibration traceable to a reference standard
  - Program the thermal cycler using the PCR Cycling Parameters in Section 8 below.

  Note: For specific thermal cycler information refer to the manufacturer's user manual. Thermal cycler should be calibrated according to ASHI (American Society of Histocompatibility and Immunogenetics) or EFI (European Federation of Immunogenetics) accreditation rules.

- Gel electrophoresis reagents (agarose, 0.5x TBE, 1000bp DNA molecular weight marker, 10mg/ml Ethidium Bromide).
- Gel electrophoresis equipment (gel tanks, power supply, gel documentation system with UV transilluminator).
- Software to assist in the manual analysis of SSPGo kit test results and the archival data storage can be downloaded from the Biofortuna website www.biofortuna.com.

**Note: any change in the specified conditions, such as thermal cycler ramp rates, may affect interpretation of the test results.**

## 6. Safety and Warnings

- For *in vitro* diagnostic use.
- Tests should only be carried out by appropriately trained personnel.
- All typing results should be verified by qualified personnel and if used for a clinical decision the results should be confirmed using another typing method.
- Handle all reagents in accordance with Good Laboratory Practice.
- Keep pre- and post-PCR areas separate. Do not bring any post-PCR materials back to the pre-PCR area.
- **Biohazard Warning:** Treat all blood products as potentially infectious.
- **Biohazard Warning:** Ethidium Bromide is a potential carcinogen. If used, always wear gloves, a laboratory coat and protective eye glasses.
- **Biohazard Warning:** Take care when using UV sources - always wear gloves, a laboratory coat and protective eye glasses. Never view the UV light source directly.
- Material Safety Data Sheets are available from www.biofortuna.com.

## 7. Storage and Stability

Biofortuna SSPGo kits should be stored at 2-28°C. Once PCR vessels are removed from the foil pouches the reagents should be re-hydrated with DNA within 3 hours. Refer to packaging for expiration date. Do not use products after the printed date.

Do not use kits if the foil pouch is ripped or perforated or if there is no desiccant bag present.

Using the sealing sheets or caps provided only, ensure PCR vessels are sealed tightly after adding DNA as omitting this may lead to evaporation during PCR amplification. Pay particular attention to edges and corners.

Note (1): If necessary, the out-of-pouch non-hydrated PCR plates and strips may be held for up to 3 hours prior to addition of DNA at a temperature of up to 21°C and humidity of no more than 60%.

Note (2): Once hydrated with DNA, PCR strips and plates from freshly opened pouches can be stored for up to 24 hours at 2–8°C before the PCR step provided that the wells are well sealed to avoid evaporation.

## 8. Directions for Use

**DNA Sample Requirements**

Each reaction in the test is optimised to utilise between 50 - 100ng of DNA, but it is critical that each reaction should be re-hydrated with exactly 10µl of liquid. Therefore, the test can only be performed with 10µl of DNA at 5-10ng/µl. Dilute the DNA to the required concentration in sterile molecular grade water only.

Caution: Ensure the final DNA sample does not contain more than 2.5mM Tris/0.25mM EDTA. Only use DNA extracted from citrate and EDTA collected samples. As heparin may inhibit PCR it is recommended that DNA should not be extracted from heparinised blood samples. Haemoglobin has been shown to interfere with SSPGo HLA kits when present in DNA samples at greater than 1 mg/dL.

DNA can be extracted using all of the traditional extraction methods. Please ensure that the $OD_{260/280}$ of the DNA sample falls between 1.66 and 1.94 as measured by UV spectrophotometry.

**Pre-PCR Directions**

i. Remove an SSPGo plate or strip from a sealed pouch.
ii. Note the product lot number of the assay.
iii. Ensure all the freeze dried pellets (PCR reagents) are at the bottom of the plate /tube wells prior to removing the seal or cap. If not, gently tap to get the pellet to the bottom of the tube.

Note that the first reaction of each test locus is always pale pink in colour to the rest of the kit. (Red when hydrated).Some PCR plates contain a purple coloured integral 'no template control' reaction in the last well of the plate.

iv. Using sterile equipment pipette 10µl DNA solution into each reaction of the plate or strip. See note, in Section 8 on DNA Sample Requirements. If the plate contains a purple coloured integral 'no template control' then pipette 10µl of sample diluent (without DNA) to it. See note on No Template Control in Section 8.
v. Ensure the DNA contacts the dry reagents in each reaction prior to thermal cycling.
vi. Seal the reactions with the sealing sheet or PCR tube caps provided. Ensure the seal is as tight as possible to prevent evaporation. Pay particular attention to edges and corners.
vii. Place tray or strips directly into the thermal cycler. Ensure the vessels are fully inserted into the block and the lid is fully compressed. Failure to do so can lead to individual PCR failure due to PCR evaporation and condensation.
viii. Run PCR program (refer to PCR Parameters*)*.

RE-SUSPENSION NOTE: Once PCR wells are removed from the foil pouches the reagents should be re-hydrated with DNA promptly. See Note (1) and Note (2) for additional information.

NO TEMPLATE CONTROL NOTE: Some kits include a No Template Control (NTC) as the final reaction on the plate. This reaction contains a purple dye to distinguish it from the rest of the reactions. The NTC is designed to detect PCR contamination, or genomic DNA contamination that may be present in the water used to re-suspend your DNA. If PCR contamination is present variable size amplicon(s) will be observed. .

PCR PLATE/STRIP HEIGHT PROFILE NOTE: It is recommended that the height profile of plates and strips are equivalent when placed in the same PCR machine. Different height profiles can cause poor contact with the PCR machines heated lid. This may result in poor or failed PCR amplification.

**PCR Parameters**

The following PCR parameters should be used. Ensure ramp speeds of 1°C per second and enable the heated lid. Please refer to the thermal cycler manufacturer's user manual for full instructions for use. Thermal cyclers should be calibrated according to the American Society of Histocompatibility and Immunogenetics (ASHI) or European Federation of Immunogenetics (EFI) accreditation rules.

| | | | |
|---|---|---|---|
| Denature | 94°C | 5 minutes | |
| Denature | 96°C | 15 seconds | |
| Anneal | 66°C | 50 seconds | 10 cycles |
| Extend | 72°C | 30 seconds | |
| Denature | 96°C | 15 seconds | |
| Anneal | 64°C | 50 seconds | 20 cycles |
| Extend | 72°C | 30 seconds | |

HOLD at 15°C for no more than 72 hours before running the gels. If necessary the PCR plate can be stored at 2 - 8 °C for up to 24 hours before running gels. Always ensure that the plates are well sealed.

**Gel Electrophoresis**

These instructions apply to horizontal agarose gel electrophoresis: Prepare a 2% agarose gel in 0.5x TBE buffer. When the gel is cooled to about 60°C add ethidium bromide to a final concentration of 0.5µg/ml. Cast gel and insert microtitre format combs (e.g. 12x8 wells with 9mm spacing). Once set, remove the combs and cover gel in 0.5x TBE buffer. Load the entire PCR product in sequence on to the 2% agarose gel, noting the position of each reaction. A 1000bp ladder is recommended to aid size determination. Run gel for 20 minutes at 10V/cm.

Refer to your electrophoresis system manufacturer's instructions for use for specific equipment details. Gels should be imaged using a UV gel documentation system with UV transilluminator.

Note: Insufficient electrophoresis may lead to large amplicons above 600bp merging with control amplicon. Please ensure the electrophoresis is sufficient to visualise such amplicons. Electrophoresing too long may result in the loss of small amplicons into preceding wells on a gel.

## 9. Interpretation

SSPGo kits are designed so the results can be determined manually using interpretation tables available from www.biofortuna.com. If you have trouble accessing the website please contact your local distributor.

The interpretation tables may include lot specific notes which may be relevant to interpretation.

Affix the gel photograph to the corresponding interpretation form by matching the kit and lot numbers. Examine the gel image. Each reaction should contain a positive control band. Refer to the interpretation tables as this may be a different size in different SSPGo products. Internal control bands might appear much weaker when allele specific bands are present. If an allele specific band is present but a control band is not, this should still be considered a positive result. Ignore any bands less than 70bp as these are unincorporated primers.

Determine the positive reactions. Positive reactions are indicated by bands of the expected size, as stated in the interpretation tables. Be aware that there may be more than one product size in a given reaction – these are multiplexed reactions and are noted on the interpretation tables.

Compare positive reactions with the interpretation tables. A positive result in a reaction indicates the presence of at least one of the alleles listed against it on the interpretation table. Any given allele may be amplified in several tubes – if the allele is present there should be a positive reaction in all of the relevant reactions.


*Figure 1: Examples of positive reactions, indicated by the presence of allele specific bands and control bands (reactions 1-3); negative reactions, indicated by the presence of control bands but absence of allele specific bands (reactions 4-7); and a failed reaction, indicated by the absence of any bands (reaction 8).*

**Ensure the kit lot number is matched correctly with the lot number on the interpretation table.**

Software to assist in the manual analysis of SSPGo kit test results and the archival data storage can be downloaded from the Biofortuna website www.biofortuna.com.

## 10. Quality Assurance and Control

Each SSPGo lot is checked for quality before any product leaves Biofortuna. Samples of each kit lot are checked against a defined panel of human DNA samples to ensure correct performance. Each reaction has been validated against a minimum of 47 well characterised cell line DNA samples. Biofortuna recommend that any laboratory should internally validate any new typing products before use on clinical samples. Only fully trained and qualified personnel should perform diagnostic typing, and results should be cross checked by another trained member of staff.

## 11. Clinical data

An in vitro diagnostic device study was performed at five test centres comparing the performance of SSPGo HLA Typing Kits to the predicate device, 'One Lambda' Labtype SSO. The locus typing completed for the SSPGo trial provided test results for HLA-A, HLA-B, HLA-C, HLA-DQA1, HLA-DQB1, HLA-DRB1/3/4/5, HLA-DPB1, DPA1, and HLA-DQA1*05, DQB1*02, DQ8.

The clinical performance of the SSPGo HLA Typing Kits provided an overall 98.3 - 100% concordance to the predicate device (SSO method) with not less than 95% confidence for genotyping of Class I and II HLA alleles tested from DNA derived from whole blood at 5-10ng/µl.

Excluding three (3) confirmed non-concordant genotyping results, 100% concordance was achieved for 1,222 samples for 3 lots of each test kit across clinical test sites in the US and UK.

Site-to-site reproducibility for SSPGo HLA Typing kits was performed by three sites using a representative panel of 8 SSPGo PCR reactions against supplied DNA samples formulated at 4ng/µl and 11ng/µl, the extremes of the test kit concentration range. A total of 958/960 individual reactions distributed equally across three external sites were concordant to DNA samples resulting in an overall 99.7% concordance (0.995 LCL).

Lot-to-Lot reproducibility was performed on three lots of a representative SSPGo HLA Test kit by a single site/operator using the DNA concentration range of 5-10 ng/µL. The lot-to-lot reproducibility for SSPGo HLA Type testing was 100% concordant to predicate device for 130/130 DNA samples tested across three (3) lots of the representative SSPGo HLA Type tests for a total of 390 tests.

Concordant SSPGo HLA positive verification was obtained for Class I and Class II alleles in comparative testing with LABType SSO tested clinical samples. SSPGo HLA internal testing provided concordant results to reference DNA samples except for unavailable samples such as certain rare HLA-DPB1 alleles and rare HLA-B alleles and allele groups such as B*59:01, B*78 and B*83:01. The following rare allele groups were not confirmed. –

B*59, B*78, B*83, DPB*21:01, DPB*26:01:02, DPB*27:01, DPB*28:01, DPB*29:01, DPB*31:01, DPB*34:01, DPB*35:01:01, DPB*39:01, DPB*45:1, DPB*46:01, DPB*51:01, DPB*55:01, DPB*59:01, DPB*63:01, DPB*81:01, DPB*85:01 & DPB*105:01

## 12. References

1) Bunce M et al Tissue Antigens. 1995 Nov;46(5):355-67.
2) Saiki RK et al. Nature. 1986 Nov 13-19;324(6093):163-6.

## 13. SSPGo Troubleshooting Guide

| Problem | Probable Cause | Remedy |
|---|---|---|
| **No amplification in any reaction** | Incorrect concentration of DNA used | Measure the DNA quantity by measuring at $OD_{280}$ and ensure 50 - 100ng of DNA in total is added in a volume of 10µl per reaction. |
| | PCR inhibitors present in DNA sample | Do not use heparinised blood, Avoid DNA samples containing greater than 1 mg/dL Hemoglobin. |
| | Poor quality DNA sample used | Measure the DNA quality. The $OD_{260/280}$ ratio should be 1.66 – 1.94 by UV spectrophotometry.<br><br>Ensure that the DNA is fully re-suspended in solution before use.<br><br>Ensure that the DNA sample was diluted in molecular grade water and does not contain more than 2.5mM Tris/0.25mM EDTA |
| | Reagents not fully re-suspended | Ensure pellets are fully re-hydrated on addition of DNA. Ensure 10µl of DNA solution is used per reaction. |
| | Thermal cycler not set up correctly | Ensure that the PCR program has been entered correctly, according to the instructions for use.<br><br>Ensure that the thermal cycler's heated lid is engaged and sufficiently tightened.<br><br>Refer to the thermal cycler's instructions for use for further guidance. |
| | Electrophoresis problems | Ensure there is power to the electrophoresis tank – check the power pack and clean the electrodes.<br><br>Run the gel in 0.5X TBE buffer.<br><br>Ensure 0.5µg/ml of fresh ethidium bromide is used. Check that there is sufficient UV illumination when imaging gels.<br><br>Refer to the gel tank and power pack manufacturer's instructions for further guidance. |
| | Plates not sealed correctly. | Insufficiently sealed plates can lead to evaporation during PCR. Biofortuna supplies recommended sealing sheets in the kit. For further supplies please contact your distributor)<br><br>Ensure there is an adequate seal across all the wells. Pay particular attention to the wells close to the edges of the PCR plate or strip. |

| Problem | Probable Cause | Remedy |
|---|---|---|
| **Random drop-outs of control and/or allele specific amplicons** | Gel errors | Ensure that all of the wells have been loaded onto the gel in the correct order, and the same volume of PCR reaction was added to each one.<br><br>Calibrate pipettors as described by the manufacturer's instructions.<br><br>Check that the wells are properly formed in the gel. Take care when removing combs as it is possible to tear the bottom of the wells.<br><br>Ensure that the agarose is fully dissolved before casting the gel.<br><br>Ensure that the gel is not run too long, as smaller amplicons may run off the end.<br><br>Ensue the gel has ran long enough to allow bands to separate.<br><br>Use fresh ethidium bromide solution. |
| | Thermal cycler problems | Failures, particularly around the edge of the assay may be due to not tightening the lid sufficiently. This can lead to evaporation and condensation of the PCR reaction half way up the PCR well and can lead to PCR failure.<br><br>Be sure to follow the manufacturer's guidance for the maintenance and calibration of your thermal cycler.<br><br>Check the PCR parameters are correct, according to the instructions for use. |
| | Evaporation problems | Ensure there is an adequate seal across all the wells. Pay particular attention to the wells close to the edges of the PCR plate or strips.<br><br>Ensure the heated lid is enabled and sufficient compression is applied via the lid. Ensure Biofortuna sealing sheets (supplied) are used. For further supplies please contact your distributor) |
| | Sporadic failure due to DNA problems | No DNA present: Ensure DNA is present in all wells.<br><br>Wrong volume: Ensure 10µl of DNA solution is added to each reaction.<br><br>Too much DNA added: Concentration of above 200ng or less than 20ng may cause PCR failure.<br><br>Contaminants in DNA may lead to sporadic or widespread failure to amplify. |

| Problem | Probable Cause | Remedy |
|---|---|---|
| **Smeary gel image** | DNA | Check the concentration and purity of the DNA. Adding too much DNA to the PCR reactions can result in smeary gel images.<br><br>Degraded or low purity can be the cause. Obtain a fresh sample of DNA |
| **Weak amplification** | DNA concentration problem | Check the DNA concentration is neither too high nor low. Aim for 50 - 100ng of DNA per reaction, in 10µl. |
| | Thermal cycler problems | Be sure to follow the manufacturer's guidance for the maintenance and calibration of your thermal cycler.<br><br>Check the PCR parameters are correct, according to the instructions for use. |
| | Gel errors | Ensure that the same volume of reaction was added to each well, between 5µl and 10µl.<br><br>Calibrate pipettors as described by the manufacturer's instructions.<br><br>Use fresh ethidium bromide solution. |
| **Non-specific amplification** | DNA concentration problem | Check the DNA concentration is neither too high nor low. Aim for between 50 - 100ng of DNA per reaction, in 10µl. |
| | Reactions loaded in the incorrect order | Check alignment of PCR and gel lanes.<br><br>Prevent physical overflow from adjacent wells in electrophoresis by not overloading and making sure gel is set before removing combs. |
| | New allele identified | Previously un-sequenced alleles may be present with a new amplification pattern. If using old interpretation sheets then download a more current alignment update from www.biofortuna.com. If this does not accommodate the new pattern you should check by using a different Biofortuna kit, or attempt to identify the sequence by sequence-based typing. |
| **Amplification pattern is not interpretable** | Incorrect interpretation of an artefact as a specific band | Check the specific Interpretation Tables for correct band size.<br><br>Check if all specific amplifications are correct in size or if an artefact (carry-over, primer dimer) has been misinterpreted as an amplification. |
| | Reactions loaded in the incorrect order | Check alignment of PCR and gel lanes. |
| | Individual PCR failure | Check all internal positive controls are present. Re-interpret without any missing reactions. |
| | Small amplicons missing | Electrophoresed too far, small amplicons have run off the end of the gel, or past the ethidium bromide front, or are dispersed by entering preceding gel well. Use |

| Problem | Probable Cause | Remedy |
|---|---|---|
| | | electrophoresis conditions suitable for your gel system. |
| | New allele identified in sample | New alleles may occasionally be discovered that may give rise to an amplification pattern that does not correspond to an existing allele(s). Please contact your local distributor. |

## 14. Revision History

**Revision: From version 4 to revision 5**

**Revision Date: 21st January 2014**

| Section | Revision Description |
|---|---|
| Section 4: Kit contents | Addition of plate seals within kits |
| Section 5: Reagents and Equipment Not Supplied | Updated 96 well thermal cycler specifications |
| | Kit storage condition updated to 2-28°C |
| | Handling of non-hydrated PCR plates and strips |
| | Handling of hydrated PCR plates and strips |
| Section 8: Directions for Use | Dilution of DNA in sterile molecular grade water |
| | Addition to interfering substances and update of DNA purity guidelines |
| | Updated $OD_{260/280}$ measurement |
| | Addition of freeze dried pellet position prior to adding sample |
| | Addition of amplified product storage conditions |
| | Addition of note regarding gel electrophoresis times |
| Section 9: Interpretation | Comment regarding batch specific notes |
| | Kit lot number to be matched correctly with the lot number on the interpretation table. |
| Section 11: Clinical Data | Addition of this complete section |
| Section 14: Revision History | Addition of this complete section |

## 15. Guide to Symbols Used

| | |
|---|---|
| Σ | Number of Tests |
| EC REP | EC Representative |
| | Consult Instructions for Use |
| | Site of Manufacture |
| IVD | In Vitro Diagnostic |
| | Expiry Date YYYY-MM-DD |
| 2°C 28°C | Storage Temperature |
| LOT | Lot Number |
| | Distributed by |
| GTIN | Global Trade Item Number |

## 16. Manufacturer Contact Details

Biofortuna Ltd
1 Hawkshead Road
Croft Business Park
Bromborough, CH62 3RJ, UK
T: +44 (0) 151 334 0182
E: info@biofortuna.com
W: www.biofortuna.com

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## 18. Translations

| | |
|---|---|
| FranÇaise: | Traductions disponibles |
| Deutsch: | Übersetzungen verfügbar |
| Español: | Traducciones disponibles |
| Italiano: | Traduzioni disponibili |
| České: | Překlady k dispozici |
| Danske: | Tilgængelige oversættelser |
| Έλληνες: | διαθέσιμες μεταφράσεις |
| Magyar: | Fordítások |
| Norske: | Oversettelser tilgjengelig |
| Polska: | Dostępne tłumaczenia |
| Português: | Traduções disponíveis |
| Россию: | Переводы доступны |
| Slovenskému: | Preklady k dispozícii |
| Türk: | Çeviriler mevcut |
| Svenska: | Översättningar tillgängliga |

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